

## DESCRIPTION

### METHOD OF JUDGING BIOLOGICAL ACTIVITY IN BIOREMEDIATION SITE AND POLYNUCLEOTIDE FOR 5 DETECTING MICROORGANISM TO BE USED THEREIN

#### Technical Field

[0001] The present invention relates to a method of judging a biological  
activity at a bioremediation site and a polynucleotide for detecting a  
10 microorganism used therein.

#### Background Art

[0002] Contamination of groundwater and soil with various types of  
organochlorine compounds including tetrachloroethylene (PCE) and  
trichloroethylene (TCE) has been a serious issue around the globe. This  
15 issue often has been covered thoroughly by mass media such as newspapers  
and the like, leading to a strong social demand for development of  
technologies for remedying environments contaminated with these  
substances.

[0003] Such technologies for remedying a contaminated environment include  
20 a physicochemical method and a biological method. Among these methods,  
particularly suited for remediation with respect to low-level contamination is  
the biological method of remedying an environment using a microorganism  
(bioremediation). Bioremediation is widely expected to be put to practical  
use since it can be performed at low cost without requiring soil excavation,  
25 allows even an environment under a building to be remedied easily, and  
achieves a reduction in environmental burdens.

[0004] Bioremediation is performed in the following manners. That is, for  
example, various nutritive substances or the like are supplied to  
microorganisms originally inhabiting contaminated soil or groundwater so  
30 that the capability of the microorganisms to degrade and eliminate

environmental contaminants is enhanced (biostimulation). Alternatively, microorganisms having the capability to degrade and eliminate environmental contaminants are introduced directly into a contaminated environment (bioaugmentation: JP 2003-154332 A, for example). An  
5 example also is known in which biostimulation and bioaugmentation were used to remedy an environment that was groundwater contaminated with TCE, yielding excellent results.

[0005] In implementing bioremediation, it is judged whether a contaminated site can be treated with biostimulation or should be treated with  
10 bioaugmentation in which microorganisms having the capability to degrade contaminants are introduced from outside of a system, and it has been desired that such judgment be performed promptly (JP 2000-079000 A, for example).

#### Disclosure of Invention

##### 15 Problem to be Solved by the Invention

[0006] With the foregoing in mind, it is an object of the present invention to provide a method whereby a microorganism in an environment contaminated with tetrachloroethylene (PCE) and trichloroethylene (TCE) is detected and the capability of the environment to degrade contaminants (a biological  
20 activity) can be judged promptly, and a polynucleotide for detecting a microorganism used in the judging method.

##### Means for Solving Problem

[0007] In order to achieve the above-described object, a method of judging a biological activity in an environment according to the present invention is a  
25 method of judging a biological activity in an environment contaminated with an organochlorine compound that is at least one of PCE and TCE. The method includes: amplifying a nucleic acid extracted from an environmental sample by a gene amplification method so as to use the nucleic acid as a target; hybridizing the target to at least one DNA probe including a base  
30 sequence unique to each of at least one type of bacterium related to

degradation of the organochlorine compound so that the at least one type of bacterium in the environment is detected; and judging the capability of the environment to eliminate the organochlorine compound based on the degrading capability of the at least one type of bacterium that is detected  
 5 with respect to the organochlorine compound and a dechlorinated product of the organochlorine compound.

The at least one DNA probe includes one or more DNA probes containing a polynucleotide according to the present invention, i.e. any one of the types of polynucleotides described below in (1) to (4).

- 10 (1) A polynucleotide comprising any one of base sequences represented by SEQ ID NOS: 1 to 17 and SEQ ID NOS: 19 to 105 of the Sequence Listing, respectively.
- (2) A polynucleotide comprising a base sequence obtained by deletion, substitution or insertion of one to several bases in the base sequence of the  
 15 polynucleotide described in (1), which is hybridizable to a polynucleotide comprising a base sequence complementary to the polynucleotide described in (1) under a stringent condition.
- (3) A polynucleotide comprising a base sequence obtained by deletion, substitution or insertion of one to several bases in the base sequence of the  
 20 polynucleotide described in (1), which has a homology of 90% or higher with the polynucleotide described in (1).
- (4) A polynucleotide comprising a base sequence complementary to any one of the polynucleotides described in (1) to (3).

Furthermore, the at least one type of bacterium related to  
 25 degradation of the organochlorine compound that is to be detected in the present invention includes one or more types of anaerobic bacteria selected from a group consisting of types of bacteria denoted below as A to R.

A: Dehalospirillum multivorans

B: Desulfitobacterium frappieri

30 C: Actinomycetales Sm-1 (Rhodococcus sp. Sm-1)

D: Rhodococcus rhodococcus

E: Xanthobacter flavus

F: Mycobacterium L1

G: Desulfomicrobium norvegicum (Desulfovibrio baculatus)

5 H: Desulfitobacterium dehalogenans

I: Desulfitobacterium hafniense

J: Clostridium formicoaceticum

K: Desulfuromonas chloroethenica

L: Acetobacterium woodii DSM 1030

10 M: Dehalobacter restrictus

N: Desulfitobacterium sp. strain PCE1

O: Desulfitobacterium frappieri TCE1

P: Acetobacterium woodii DSM 2396

Q: Desulfomonile tiedjei DCB-1

15 R: Dehalococcoides ethenogenes 195

Effects of the Invention

[0008] In relation to bioremediation with respect to an environment contaminated with an organochlorine compound such as PCE, TCE or the like, if an anaerobic bacterium capable of degrading the organochlorine

20 compound and a dechlorinated product thereof can be detected promptly from the contaminated environment, an assessment of whether biostimulation can be performed or bioaugmentation should be performed is facilitated. The inventors of the present invention took note of this fact and conducted vigorous studies on a method of detecting the currently known 18 types of  
25 anaerobic bacteria capable of degrading organochlorine compounds and dechlorinated products thereof (the types of bacteria denoted as A to R; hereinafter, referred to also as anaerobic bacteria related to degradation of organochlorine compounds).

[0009] As a result of the studies, the inventors found that each of the types  
30 of bacteria denoted as A to R has specificity in terms of base sequences in an

ITS region (SEQ ID NOS: 1 to 18 of the Sequence Listing) that is a genome region common to the types of bacteria denoted as A to R, and the use of a DNA probe containing a polynucleotide based on the base sequences allows each of the types of bacteria denoted as A to R to be detected using a genetic detection technique such as, for example, a DNA microarray.

[0010] The inventors of the present invention conducted further studies to determine, based on the base sequences in the respective ITS regions of the 18 types of bacteria denoted as A to R (SEQ ID NOS: 1 to 18 of the Sequence Listing), specific base sequences of a length that can be used suitably for, for example, a DNA microarray, with which the types of bacteria denoted as A to R can be detected at the same time without cross-reacting with one another (SEQ ID NOS: 19 to 115 of the Sequence Listing), thus arriving at the present invention.

[0011] The ITS region refers to a transcribed region between a 16S ribosomal DNA and a 23S ribosomal DNA of a bacteria genome occurs (Internal Transcribed Spacer). The base sequences in the respective ITS regions of the types of bacteria denoted as A to Q (assigned SEQ ID NOS: 1 to 17, respectively) were determined for the first time by the inventors of the present invention.

[0012] According to the present invention, a bacterium related to degradation of an organochlorine compound in a contaminated environment is detected promptly using a DNA probe, and based on the degrading capability of the bacterium (see, for example, FIG. 5B), the capability of the environment to eliminate PCE and a dechlorinated product thereof can be judged. Therefore, according to the present invention, in implementing bioremediation, an assessment of whether biostimulation can be performed or bioaugmentation should be performed is facilitated, thereby allowing the selection of a proper method of remedying an environment. Moreover, since a method of remedying an environment is selected promptly and properly, remediation of the environment can be performed at low cost.

[0013] It is thought that PCE hardly is degraded under an aerobic condition. Further, generally, an environment 50 cm or more below the surface of the Earth is believed to be anaerobic. Therefore, in remedying an environment contaminated with PCE or a contaminated environment 50 cm or more below  
5 the surface of the Earth, the use of an aerobic microorganism instead of an anaerobic microorganism that essentially is applicable may incur extra cost and energy consumption. The present invention can avoid incurring such extra cost and energy use.

#### Brief Description of Drawings

10 [0014] [FIG. 1] FIG. 1 is a diagram showing results of detection using a DNA microarray performed in Example 1.

[FIG. 2A] FIG. 2A is a photograph showing results of scanning a DNA microarray in Example 2.

15 [FIG. 2B] FIG. 2B is a graph showing a result of detecting a bacterium M using the DNA microarray in Example 2.

[FIG. 3A] FIG. 3A is a photograph showing results of scanning a DNA microarray in Example 3.

[FIG. 3B] FIG. 3B is a graph showing a result of detecting a bacterium J using the DNA microarray in Example 3.

20 [FIG. 4] FIG. 4 shows photographs showing results of scanning a DNA microarray in Example 4, in which bacteria A, B, I, J, M, N and O were detected, respectively.

[FIG. 5A] FIG. 5A is a diagram illustrating a degradation route of PCE.

25 [FIG. 5B] FIG. 5B is a diagram explaining degradation activities of bacteria A to R with respect to organochlorine compounds.

#### Description of the Invention

[0015] Bacteria to be detected in the biological activity judging method according to the present invention are bacteria related to degradation of  
30 organochlorine compounds and include one or more types of bacteria among

the 18 types denoted as A to R that are currently known to be involved in degradation of PCE into ethene or carbon dioxide. Furthermore, the bacteria related to degradation of organochlorine compounds are not limited thereto and include bacteria that will be identified in the future to be involved in degradation of PCE.

[0016] The types of bacteria denoted as A to Q are assigned the following deposit numbers by either of ATCC and DSMZ that are organizations for conservation of living resources and are available from the corresponding one of the organizations.

A: DSM 12446	B: DSM 13498	C: ATCC 51239
D: ATCC 21197	E: DSM 10330	F: DSM 6695
G: DSM 1741	H: DSM 9161	I: DSM 10644
J: ATCC 27076	K: DSM 12431	L: DSM 1030
M: DSM 9455	N: DSM 10344	O: DSM 12704
P: DSM 2396	Q: ATCC 49306	

[0017] PCE is dechlorinated to TCE, then to dichloroethylene (DCE), and further to vinyl chloride (VC), and in a final stage, degraded into ethene or carbon dioxide. FIG. 5A shows a typical degradation route of PCE.

Degradation activities of the types of bacteria denoted as A to R with respect to PCE and dechlorinated products thereof have already been known, and the respective biological activities of these types of bacteria are shown in FIG. 5B. Thus, if at least one of the types of bacteria denoted as A to R can be detected in an environment, it can be judged that the environment is provided with a biological activity corresponding to a degradation activity of the at least one of the types of bacteria thus detected.

[0018] Concretely, for example, in the case where at least one of the types of bacteria denoted as J, L and P is detected in an environment, it can be judged that the environment has a biological activity of degrading PCE into TCE.

Further, in the case where at least one of the types of bacteria denoted as A, G and M is detected, it can be judged that the environment has a biological activity of degrading PCE into cis-dichloroethylene (cisDCE). Further, in the case where at least one of the types of bacteria denoted as B, I, H, N, O and Q

is detected, it can be judged that the environment has a biological activity of degrading PCE and TCE into cisDCE. Further, in the case where the bacterium K is detected, it can be judged that the environment has a biological activity of degrading PCE and TCE into DCE. Further, in the case  
5 where the bacterium R is detected, it can be judged that the environment has a biological activity of degrading PCE, TCE, DCE and VC into ethene. Further, in the case where at least one of the types of bacteria denoted as C, D and E is detected, it can be judged that the environment has a biological activity of degrading DCE and VC into carbon dioxide. Further, in the case  
10 where the bacterium F is detected, it can be judged that the environment has a biological activity of degrading VC into carbon dioxide. Moreover, in the case where two or more types of bacteria that have different degradation activities are detected, it can be judged that the environment has a biological activity as a combination of the respective degradation activities.

15 [0019] A method of detecting a bacterium in the biological activity judging method according to the present invention includes steps of: extracting a nucleic acid from an environmental sample and producing a target by a gene amplification method; and hybridizing the target to a DNA probe specific to a bacteria to be detected.

20 [0020] In the present invention, the DNA probe is derived from an ITS region of a bacterium to be detected. That is, a polynucleotide entirely or partly including one of the base sequences represented by SEQ ID NOS: 1 to 18 of the Sequence Listing can be used as the DNA probe in the present invention.

25 [0021] Generally, a 16SrRNA, a 23SrRNA and a 5SrRNA that are ribosomal RNAs (rRNAs) of prokaryotes are transcribed as one transcription unit (operon), and therefore, a 16SrRNA gene and a 23 SrRNA gene are located adjacently on a genome. A region between these genes, i.e. a 16SrRNA gene and a 23SrRNA gene is a so-called 16S–23S Internal Transcribed Spacer  
30 (ITS) region.



[0022] The inventors of the present invention determined, with respect to the types of bacteria denoted as A to Q, base sequences in the respective ITS regions (assigned SEQ ID NOS: 1 to 17 of the Sequence Listing, respectively) and found for the first time that a polynucleotide having a base sequence  
5 within each of these ITS regions can be used to produce a DNA probe unique to each of the types of bacteria denoted as A to Q. The genome base sequence of the bacterium R is determined by Dr. Zinder of Cornell University. Since the base sequence in an ITS region of the bacterium R (SEQ ID NO: 18 of the Sequence Listing) also is specific to the bacterium R, when a DNA  
10 probe derived from the ITS region of the bacterium R is used in combination with DNA probes derived respectively from the ITS regions of the 17 types of bacteria denoted as A to Q, the DNA probes can be used as a group of DNA probes that allows all the 18 types of bacteria denoted as A to R to be detected.

15 [0023] As a DNA probe for detecting the types of bacteria denoted as A to R, a DNA probe including a part of an ITS sequence is used more preferably than a DNA probe including the entire ITS sequence. This is because, in general, the sequence specificity of a DNA probe increases with decreasing length of the DNA probe, thus providing improved reliability. Meanwhile, it  
20 is necessary that a sequence itself is unique to each of the types of bacteria. A DNA probe has a length of, for example, 10 bases to the entire ITS sequence, and preferably 40 to 80 bases, though there is no limitation thereto.

[0024] As a DNA probe for detecting the types of bacteria denoted as A to R, a polynucleotide can be used that is derived from an ITS region and has a  
25 length of 40 bases, and a concrete example of such a polynucleotide comprises base sequences represented by SEQ ID NOS: 19 to 115 of the Sequence Listing.

[0025] That is, a polynucleotide including one of the base sequences represented by SEQ ID NOS: 19 to 25 of the Sequence Listing can be used as  
30 a DNA probe for detecting the bacterium A; a polynucleotide including one of

the base sequences represented by SEQ ID NOS: 26 to 30 of the Sequence Listing can be used as a DNA probe for detecting the bacterium B; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 31 to 35 of the Sequence Listing can be used as a DNA probe for

5 detecting the bacterium C; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 36 to 40 of the Sequence Listing can be used as a DNA probe for detecting the bacterium D; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 41 to 45 of the Sequence Listing can be used as a DNA probe for detecting the bacterium

10 E; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 46 to 48 of the Sequence Listing can be used as a DNA probe for detecting the bacterium F; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 49 to 53 of the Sequence Listing can be used as a DNA probe for detecting the bacterium G; a polynucleotide

15 including one of the base sequences represented by SEQ ID NOS: 54 to 57 of the Sequence Listing can be used as a DNA probe for detecting the bacterium H; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 58 to 62 of the Sequence Listing can be used as a DNA probe for detecting the bacterium I; a polynucleotide including one of the base

20 sequences represented by SEQ ID NOS: 63 to 68 of the Sequence Listing can be used as a DNA probe for detecting the bacterium J; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 69 to 74 of the Sequence Listing can be used as a DNA probe for detecting the bacterium K; a polynucleotide including one of the base sequences represented by SEQ

25 ID NOS: 75 to 79 of the Sequence Listing can be used as a DNA probe for detecting the bacterium L; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 80 to 86 of the Sequence Listing can be used as a DNA probe for detecting the bacterium M; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 87 to 91 of

30 the Sequence Listing can be used as a DNA probe for detecting the bacterium

N; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 92 to 96 of the Sequence Listing can be used as a DNA probe for detecting the bacterium O; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 97 to 99 of the Sequence Listing can be used as a DNA probe for detecting the bacterium P; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 100 to 105 of the Sequence Listing can be used as a DNA probe for detecting the bacterium Q; a polynucleotide including one of the base sequences represented by SEQ ID NOS: 106 to 115 of the Sequence Listing can be used as a DNA probe for detecting the bacterium R.

[0026] With respect to each of the above-described types of polynucleotides comprising the base sequences SEQ ID NOS: 1 to 115 of the Sequence Listing, respectively, even a polynucleotide comprising a base sequence obtained by deletion, substitution or insertion of one to several bases therein can be used as a DNA probe for the biological activity judging method according to the present invention. That is, the DNA probe according to the present invention is a probe containing any one of types of polynucleotides described below in (1) to (4).

(1) A polynucleotide comprising any one of the base sequences represented by SEQ ID NOS: 1 to 115 of the Sequence Listing, respectively.

(2) A polynucleotide comprising a base sequence obtained by deletion, substitution or insertion of one to several bases in the base sequence of the polynucleotide described in (1), which is hybridizable to a polynucleotide comprising a base sequence complementary to the polynucleotide described in (1) under a stringent condition.

(3) A polynucleotide comprising a base sequence obtained by deletion, substitution or insertion of one to several bases in the base sequence of the polynucleotide described in (1), which has a homology of 90% or higher with the polynucleotide described in (1).

(4) A polynucleotide comprising a base sequence complementary to any one of

the polynucleotides described in (1) to (3).

[0027] The number of bases that can be deleted, substituted or inserted is, for example, as follows. That is, with respect to 40 bases, the number of bases that can be deleted/inserted is 1 to 6, preferably 1 to 3, and more preferably 1 to 2, and the number of bases that can be substituted is 1 to 4, preferably 1 to 2, and more preferably 1. Further, hybridization under a stringent condition means that two DNA fragments are hybridized to each other under a standard hybridization condition as described by Sambrook J. et al. (Expression of cloned genes in *E. coli* (Molecular Cloning: A laboratory manual (1989)) Cold Spring harbor Laboratory Press, New York, USA, 9. 47-9. 62 and 11.45-11.61). More concretely, it means that hybridization and washing (for example, in about  $2.0 \times \text{SSC}$  at  $50^\circ\text{C}$ ) are performed with reference to  $\pm 10^\circ\text{C}$  of a  $T_m$  value. Further, the homology is, for example, 90% or higher, preferably 95% or higher, and more preferably 97.5% or higher.

[0028] A gene amplification method for preparing a target to be hybridized to the DNA probe is not limited particularly as long as an ITS region of a bacterium to be detected can be amplified by the method, and as the method, for example, a PCR method can be employed. Since the DNA probe in the present invention is derived from an ITS region of a bacterium, for example, in one gene amplification reaction using a combination of a small number of primers, ITS regions as targets of all the types of bacteria to be detected can be amplified.

[0029] For example, the ITS regions of the types of bacteria denoted as A to Q can be amplified by the use of a polynucleotide comprising a base sequence represented by SEQ ID NO: 116 of the Sequence Listing as a sense primer and a polynucleotide comprising a base sequence represented by SEQ ID NO: 117 of the Sequence Listing as an antisense primer. Moreover, in the case of amplifying the ITS region of the bacterium R as well at the same time, a polynucleotide comprising a base sequence represented by SEQ ID NO: 118 of

the Sequence Listing as an antisense primer further should be added to a reaction solution.

[0030] A detection method by hybridization of a target amplified in the above-described manner to the DNA probe is not limited particularly, and as  
5 the method, for example, a conventionally known gene detection technique such as a Southern blotting method, a DNA array method, a DNA microarray method, or a DNA chip method can be used. Among these, a method in which a DNA probe corresponding to a bacterium to be detected is immobilized allows bacteria in all environmental samples to be detected at  
10 one time and thus is preferable. Preferably, the target and the DNA probe are labeled suitably depending on the detection method. For example, the target may be labeled at the same time that gene amplification for preparing the target is performed. There is no particular limitation to the labeling, and, for example, fluorescence labeling or RI labeling can be employed.

[0031] An environment as a subject of biological activity judgment according to the present invention is not limited particularly and can be, for example, soil, groundwater, pond water, or seawater that is contaminated with at least one of PCE and TCE. A nucleic acid to be used as a template of the target can be extracted from the environmental sample by a conventionally known  
20 method such as, for example, using a commercially available nucleic acid extracting kit without any particular limitation. As the nucleic acid, for example, a DNA or a RNA may be used.

[0032] A bioremediation method according to the present invention is a method of bioremediation with respect to an environment contaminated with  
25 an organochlorine compound that is at least one of PCE and TCE, and includes steps of: performing the method of judging a biological activity in an environment according to the present invention; and stimulating, when a bacterium related to degradation of the organochlorine compound is detected by the method, growth and/or an activity of the bacterium so as to enhance  
30 the degradation of the organochlorine compound or a dechlorinated product

thereof. In the bioremediation, the capability of an environment to degrade and eliminate an organochlorine compound such as PCE or the like is grasped beforehand by the biological activity judging method according to the present invention, and thus it becomes possible to select a bioremediation method so that the accuracy and promptness of bioremediation can be increased. For example, if the bacterium R is detected, it is expected that PCE can be degraded into ethene using the bacterium R, and thus based on this, biostimulation in which nutrients for enhancing growth and an activity of the bacterium R is introduced into an environment can be selected.

Further, for example, similarly in the case where the bacteria K and C are detected, it is expected that PCE can be degraded into carbon dioxide using the two types of bacteria (see FIG. 5B), and thus based on this, biostimulation in which nutrients for enhancing growth and activities of the two types of bacteria are introduced into an environment can be selected.

[0033] A bioremediation method according to another aspect of the present invention is a method of bioremediation with respect to an environment contaminated with an organochlorine compound that is at least one of PCE and TCE, and includes steps of: performing the method of judging a biological activity in an environment according to the present invention; and adding at least one of types of bacteria related to degradation of the organochlorine compound other than a detected bacterium to the environment so as to enhance the degradation of the organochlorine compound or a dechlorinated product thereof. For example, in the case where only the bacterium K is detected, the addition of the bacterium C to the environment allows PCE to be degraded into carbon dioxide.

[0034] A device for detecting a bacterium according to the present invention is a device that can be used in the biological activity judging method according to the present invention, and includes the DNA probe according to the present invention. The detection device according to the present invention is not limited particularly as long as the device includes the DNA

probe that is immobilized and can detect the target hybridized to the DNA probe. In the detection device according to the present invention, preferably, at least two such DNA probes are included, and at least two of the above-described types of bacteria can be detected at the same time. More preferably, the detection device includes a polynucleotide including one of the base sequences represented by SEQ ID NOS: 19 to 115 of the Sequence Listing as a DNA probe and can detect all of the types of bacteria denoted as A to R at the same time.

[0035] A DNA microarray according to the present invention is a DNA microarray that can be used in the biological activity judging method according to the present invention, and includes a substrate on which at least one DNA probe according to the present invention is immobilized. In the DNA microarray according to the present invention, preferably, two or more DNA probes according to the present invention are immobilized, and two or more of the above-described types of bacteria can be detected. In order to suppress noise, preferably, the length of the DNA probe(s) immobilized to the DNA microarray according to the present invention is as short as possible, and in order to suppress cross-hybridization by keeping a  $T_m$  value constant, preferably, the DNA probes have the same length. There is no particular limitation to the substrate used in the DNA microarray according to the present invention, and as the substrate, a commercially available substrate for a DNA microarray or the like can be used, and the DNA probe(s) can be immobilized to the substrate by a conventionally known method with no particular limitation.

[0036] A detecting kit according to the present invention is a kit for detecting a bacterium that can be used in the biological activity judging method according to the present invention, and includes: the DNA probe according to the present invention; and a primer for gene amplification and a reagent for gene amplification that are used for preparing a target to be hybridized to the DNA probe so as to be detected. A kit according to another aspect of the

present invention includes the DNA microarray according to the present invention instead of the DNA probe according to the present invention. As the primer for gene amplification, for example, a polynucleotide including the base sequence represented by SEQ ID NO: 116 of the Sequence Listing can be  
5 used as a sense primer, and a polynucleotide(s) including the base sequence(s) represented by SEQ ID NO(S): 117 and/or 118 of the Sequence Listing can be used as a antisense primer. As the reagent for gene amplification, a conventionally known reagent such as, for example, a buffer, polymerase, or nucleotide can be used. When necessary, the detection kit according to the  
10 present invention may include, for example, a reagent for extracting a nucleic acid and a filter or a chip that are used for preparing a target.

[0037] A polynucleotide according to the present invention is a new type of polynucleotide that can be used as a DNA probe for detecting the types of bacteria denoted as A to Q in the biological activity judging method according  
15 to the present invention, and is any one of types of polynucleotides described below in (1) to (4). The number of bases to be deleted, substituted or inserted, a stringent condition and a homology are defined in the same manner as in the foregoing description.

(1) A polynucleotide comprising any one of base sequences represented by  
20 SEQ ID NOS: 1 to 17 and SEQ ID NOS: 19 to 105 of the Sequence Listing, respectively.

(2) A polynucleotide comprising a base sequence obtained by deletion, substitution or insertion of one to several bases in the base sequence of the polynucleotide described in (1), which is hybridizable to a polynucleotide  
25 comprising a base sequence complementary to the polynucleotide described in (1) under a stringent condition.

(3) A polynucleotide comprising a base sequence obtained by deletion, substitution or insertion of one to several bases in the base sequence of the polynucleotide described in (1), which has a homology of 90% or higher with  
30 the polynucleotide described in (1).



(4) A polynucleotide comprising a base sequence complementary to any one of the polynucleotides described in (1) to (3).

[0038] Hereinafter, the present invention will be described by way of examples.

## 5 Examples

### Example 1

[0039] (Production of a DNA probe and a DNA microarray that allow 18 types of bacteria to be detected at the same time)

#### 1. Production of DNA probe

10 A base sequence having 40 bases was designed based on the ITS sequences of the types of bacteria denoted as A to R (assigned SEQ ID NOS: 1 to 18 of the Sequence Listing, respectively) and used as a DNA probe. In designing the DNA probe, a precondition was set that the DNA probe was a single-stranded probe of 40 bases having a GC content of 48 to 50%, exhibited  
15 no or almost no complementation, and was not found in the International Nucleotide Sequence Database GenBank (if any, contained two or more mispairs). The following are DNA probes thus produced (of 3 to 10 types for each type of bacterium) and sequence ID numbers of their respective base sequences (SEQ ID NOS: 19 to 115 of the Sequence Listing).

20 [0040] Probes A1 to A7 for the bacterium A have the base sequences represented by SEQ ID NOS: 19 to 25 of the Sequence Listing, respectively; probes B1 to B5 for the bacterium B have the base sequences represented by SEQ ID NOS: 26 to 30 of the Sequence Listing, respectively; probes C1 to C5 for the bacterium C have the base sequences represented by SEQ ID NOS: 31  
25 to 35 of the Sequence Listing, respectively; probes D1 to D5 for the bacterium D have the base sequences represented by SEQ ID NOS: 36 to 40 of the Sequence Listing, respectively; probes E1 to E5 for the bacterium E have the base sequences represented by SEQ ID NOS: 41 to 45 of the Sequence Listing, respectively; probes F 1 to F3 for the bacterium F have the base sequences  
30 represented by SEQ ID NOS: 46 to 48 of the Sequence Listing, respectively:

probes G1 to G5 for the bacterium G have the base sequences represented by SEQ ID NOS: 49 to 53 of the Sequence Listing, respectively; probes H1 to H4 for the bacterium H have the base sequences represented by SEQ ID NOS: 54 to 57 of the Sequence Listing, respectively; probes I1 to I5 for the bacterium I  
5 have the base sequences represented by SEQ ID NOS: 58 to 62 of the Sequence Listing, respectively; probes J1 to J6 for the bacterium J have the base sequences represented by SEQ ID NOS: 63 to 68 of the Sequence Listing, respectively; probes K1 to K6 for the bacterium K have the base sequences represented by SEQ ID NOS: 69 to 74 of the Sequence Listing, respectively;  
10 probes L1 to L5 for the bacterium L have the base sequences represented by SEQ ID NOS: 75 to 79 of the Sequence Listing, respectively; probes M1 to M7 for the bacterium M have the base sequences represented by SEQ ID NOS: 80 to 86 of the Sequence Listing, respectively; probes N1 to N5 for the bacterium N have the base sequences represented by SEQ ID NOS: 87 to 91 of the  
15 Sequence Listing, respectively; probes O1 to O5 for the bacterium O have the base sequences represented by SEQ ID NOS: 92 to 96 of the Sequence Listing, respectively; probes P1 to P3 for the bacterium P have the base sequences represented by SEQ ID NOS: 97 to 99 of the Sequence Listing, respectively; probes Q1 to Q6 for the bacterium Q have the base sequences represented by  
20 SEQ ID NOS: 100 to 105 of the Sequence Listing, respectively; probes R1 to R10 for the bacterium R have the base sequences represented by SEQ ID NOS: 106 to 115 of the Sequence Listing, respectively.

[0041]

## 2. Production of DNA microarray and confirmation of specificity of DNA 25 probe

Next, using an Affymetrix 417 Arrayer (manufactured by Affymetrix, Inc.), each of the above-described 97 types of DNA probes was printed in a customized manner on a TaKaRa-Hubble Slide Glass (manufactured by Takara Bio Inc.) to produce a DNA microarray. Then, a target was prepared  
30 using each of the types of bacteria denoted as A to R and hybridized to the

DNA microarray so as to be used to confirm the specificity of the DNA probes.

[0042] The target was prepared in a manner that an ITS region of each of the above-described types of bacteria was amplified by a PCR method. In performing the PCR, as a sense primer, a non-labeled primer having the base sequence represented by SEQ ID NO: 116 of the Sequence Listing was used. Further, as an antisense primer, a Cy3-labeled primer having the base sequence represented by SEQ ID NO: 117 of the Sequence Listing was used for the bacteria other than the bacterium R, and a Cy3-labeled primer having the base sequence represented by SEQ ID NO: 118 of the Sequence Listing was used for the bacterium R. Reaction conditions for the PCR were set so as to conform to a standard protocol.

[0043] An amplified product to be used as a target resulting from the PCR was desalted using an AutoSeq G-50 (manufactured by Pharmacia Corporation), then vacuum-dried using a SpeedVac (manufactured by Savant Instrument, Inc.) and then dissolved in a buffer of  $5 \times$  SSC, 0.2% SDS and 50% formamide at their respective final concentrations. A target solution thus obtained was boiled at a temperature of  $94^{\circ}\text{C}$  for 3 minutes, then cooled with ice for at least two minutes, and then applied on the DNA microarray. The DNA microarray was covered with a cover glass and then placed in a hybridization chamber at a set temperature of  $42^{\circ}\text{C}$  for at least 4 hours. After that, the DNA microarray was washed with  $0.2 \times$  SSC and 0.2% SDS for 5 minutes, with  $0.2 \times$  SSC for 5 minutes, and with  $0.05 \times$  SSC for several seconds, and then spin-dried at a speed of 1,800 rpm. Scanning was performed using a ScanArray version 5 (manufactured by PerkinElmer Japan Co., Ltd.) to obtain measurement results.

[0044] The results are shown in FIG. 1. FIG. 1 is a graph showing whether the targets derived from the respective ITS sequences of the 18 types of bacteria denoted as A to R plotted on a vertical axis were hybridized to the 97 DNA probes plotted on a horizontal axis. In the figure, black-painted portions show that the DNA probes were hybridized to the targets,

respectively exhibiting fluorescent signals of 500 fluorescent units or more. A phylogenetic tree on the vertical axis was created based on the alignment of the ITS sequences. As shown in FIG. 1, it was proved that the targets prepared using the types of bacteria denoted as A to R were hybridized significantly only to the DNA probes for the types of bacteria denoted as A to R, respectively, without being cross-hybridized.

#### Example 2

[0045] (Judgment 1 of biological activity in contaminated environment)

Using a FastPrep bead-beater and a soil DNA extraction kit (manufactured by Qbiogene, Inc.) and following instruction manuals, a DNA was extracted from 250 mg of a soil sample provided by Matsushita Environmental & Air-conditioning Engineering. The DNA in an amount of about 1  $\mu$ l was added to 50  $\mu$ l of a standard Amplitaq Gold PCR mixture (manufactured by Applied Biosystems) containing a non-labeled sense primer 27F (SEQ ID NO: 116 of the Sequence Listing) and a Cy3-labeled antisense primer 132 R (SEQ ID NO: 117 of the Sequence Listing) or 341R (SEQ ID NO: 118 of the Sequence Listing). After performing PCR in accordance with a standard protocol, a PCR amplified product was desalted using an Autoseq G-50 (manufactured by Pharmacia Corporation) and then vacuum-dried using a SpeedVac (manufactured by Savant Instrument, Inc.). The PCR amplified product thus dried was dissolved in a buffer of 5  $\times$  SSC, 0.2% SDS and 50% formamide at their respective final concentrations, and a solution thus obtained was boiled at a temperature of 94°C for 3 minutes, then cooled with ice for at least two minutes, and then applied on the DNA microarray produced in Example 1. The DNA microarray was covered with a cover glass and then placed in a hybridization chamber at a set temperature of 42°C for at least 4 hours. After that, the DNA microarray was washed with 0.2  $\times$  SSC and 0.2% SDS for 5 minutes, with 0.2  $\times$  SSC for 5 minutes, and with 0.05  $\times$  SSC for several seconds, and then spin-dried at a speed of 1,800 rpm. Scanning was performed using a ScanArray version 5 (manufactured by

PerkinElmer Japan Co., Ltd.) to obtain measurement results.

[0046] The results of the scanning of the DNA microarrays are shown partially in FIG. 2. FIG. 2A shows scan images, and FIG. 2B is a graph in which the results are shown quantitatively. As shown in FIG. 2, significant hybridization of the bacterium M (*Dehalobacter restrictus* DSM 945) to the probes was detected. Thus, it could be judged that the soil as the sample had capability to degrade PCE into cisDCE (See FIG. 5B).

#### Example 3

[0047] (Judgment 2 of biological activity in contaminated environment)

In this example, 300 ml of a groundwater sample provided by Matsushita Environmental & Air-conditioning Engineering was used instead of the soil sample, and a DNA was extracted from debris obtained by centrifugation at a speed of 7,000 rpm with respect to the groundwater sample. Except for this, in the same manner as in Example 2, bacteria in the groundwater sample were detected using the DNA microarray.

[0048] The results of the detection are shown partially in FIG. 3. FIG. 3A shows scan images, and FIG. 3B is a graph in which the results are shown quantitatively. As shown in FIG. 3, significant hybridization of the bacterium J (*Clostridium formicoaceticum* ATCC 27076) to the probes was detected. Thus, it could be judged that the soil as the sample had the capability to degrade PCE into TCE (See FIG. 5B).

#### Example 4

[0049] (Judgment 3 of biological activity in contaminated environment)

In this example, an anaerobic enrichment culture sample provided by Dr. T. H. Lee (the Republic of Korea) was used instead of the soil sample. Except for this, in the same manner as in Example 2, the bacteria in the sample were detected.

[0050] Results of the detection are shown in FIG. 4. As shown in FIG. 4, strong signals were detected in part of the probes for the bacteria A, J, M, N and O, and weak signals were detected also in the probes for the bacteria B

and J. Thus, it could be judged that the sample had capability to convert PCE into cisDCE (see FIG. 5B). These judgment results agreed with the analysis data on PCE/cisDCE in the enrichment culture provided by Dr. T. H. Lee.

5 Industrial Applicability

[0051] As described in the foregoing discussion, the method of judging a biological activity in an environment according to the present invention and the polynucleotide according to the present invention are useful in the method of remedying an environment contaminated with PCE or the like,  
10 particularly, in the field of bioremediation.

Sequence Listing Free Text

[0052]

SEQ ID NO: 116 Sense primer 27F for PCR

SEQ ID NO: 117 Antisense primer 132R for PCR

15 SEQ ID NO: 118 Antisense primer 341R for PCR